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14. ABSTRACT- Prostate cancer cells preferentially metastasize to bone, presumably through physical interaction with E-selectin present on human bone marrow endothelial cells. We examined the interaction between prostate cancer cell lines and circulating tumor cells (CTCs) isolated from prostate cancer patients with stimulated endothelial cells expressing E-selectin under physiological blood flow using a parallel flow chamber system. Prostate cancer cells, MDAPCa 2b were first labeled with anti-PSMA monoclonal antibody J591 conjugated with alexa fluor 488 (J591-488) that recognizes prostate specific membrane antigen (PSMA) and is internalized following binding to PSMA. We observed that the mean rolling velocity of MDAPCa 2b cells on HUVECs ranged from 4.2-6 $\mu\text{m/s}$ at 0.5-4 $\text{dyn/cm}^2$ shear stress. Interestingly, MDAPCa2b cells did not show rolling behavior on both unstimulated-HUVECs and stimulated-HUVECs incubated with anti-E-selectin neutralizing antibody ( $p < 0.005$ ) at different shear stress. We then used J591-488 antibody to label 4 metastatic prostate cancer patient CTCs and found that in 3 patients, prostate CTCs tethered and stably interacted with activated HUVECs expressing E-selectin at 0.6 $\text{dyn/cm}^2$ shear stress. Furthermore, in parallel CTC-Endothelial interactions in same patients were abrogated in the presence of anti-E-selectin neutralizing antibody. We also found the presence of sialyl-Lewis X (sLex), an epitope present on E-selectin ligands, on CTCs derived from metastatic prostate cancer patients. Fluorescence intensity analysis of sLex expression in prostate cancer patient CTCs showed a heterogeneous expression of sLex ranging from 44-75%. These findings confirmed that prostate CTCs exhibit E-selectin-dependent adhesive interactions with endothelial cells. These results highly suggest the presence of E-selectin ligands on the surface of prostate CTCs which might be eventually responsible for tumor metastasis.					
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## 1. INTRODUCTION:

Prostate cancer (PCa) is the leading cause of cancer in men and second to lung cancer in cancer-associated mortality in United States. The prognosis for 5-year survival is >90% if detected at an early stage while it decreases to 31% for patients diagnosed with metastases (1). A vast majority of metastatic prostate cancer cells home to the bone marrow endothelium. Studies suggest that metastatic tumor cells exploit similar mechanisms as used by leukocytes for adhesion and extravasation through endothelium during inflammatory response that may lead to metastasis (2,3). Selectin-Selectin ligand interactions between endothelial cells and leukocytes primarily causes a rolling behavior which subsequently leads to firm adhesion and extravasation via other adhesive proteins (4). Many investigators have been studying metastases in PCa using PCa cell lines and their interactions with various endothelial cell lines. However, these studies depend on the cell lines, frequently passaged *in vitro* for months to years and may not replicate the *in vivo* invasive characteristics critical for cancer development and metastatic progression. The goal of our study is to examine the interactions between human umbilical vein endothelial cells (HUVECs) and circulating tumor cells (CTCs) derived from prostate cancer patients. We have developed a novel method to study PCa CTCs isolated from patients with metastatic PCa by taking advantage of the fact that prostate CTCs express prostate specific membrane antigen (PSMA) on their cell surface. Through “*ex vivo*” labeling, we can track and analyze the interactions between CTCs and HUVECs, and identify the surface proteins critical to the CTC-endothelial cell interactions. The identification and characterization of the proteins present on adherent CTCs can provide targets to develop anti-metastatic therapies.

## 2. KEYWORDS:

Circulating tumor cells, E-selectin, Human umbilical vein endothelial cells, Prostate cancer, Metastasis, Prostate specific membrane antigen, Selectin-Selectin ligand interactions

## 3. OVERALL PROJECT SUMMARY:

### Current Objectives

1. *To confirm the expression of E-selectin on activated endothelial cells*
2. *To perform rolling velocity assay on endothelial cells using prostate cancer cells*
3. *To examine the interactions between endothelial cells and CTCs obtained from prostate cancer patients*

### Results

**Subtask 1a:** *Confirming the expression of induced E-selectin by HBMEC or HUVECs (1-2 months)*

Both primary and E4ORF1 HUVECs (5) were used to examine the expression of E-selectin. Both primary and E4ORF1 HUVECs showed the expression of E-selectin in the presence of 50 ng/ml IL-1 $\beta$  (Figure 1A). Unstimulated HUVECs were used as a control, which did not express E-selectin as examined by immunofluorescence (data not shown). To confirm, that both primary and E4ORF1 HUVECs expressed similar levels of E-selectin protein expression, western blotting was performed. We observed that both primary and E4ORF1 HUVECs showed similar levels of protein expression for E-

selectin (Figure 1B). Furthermore, a functional assay was performed to examine any difference in the rolling behavior between primary and E4ORF1 HUVECs. We analyzed that the average rolling velocity of MDAPCa2b cells on both primary and E4ORF1 HUVECs was 6.35 and 5.94  $\mu\text{m}/\text{sec}$ , respectively. No significant difference was observed in the rolling velocity. In conclusion, these experiments suggested that either primary or E4ORF1 HUVECs can be used for observing interactions with prostate cancer cells without any significant effect. Therefore, subsequent experiments were done using E4ORF1 HUVECs. We also tested TNF $\alpha$  for the upregulation of E-selectin expression in HUVECs; however no expression was observed. Hence, we used IL-1 $\beta$  for further experiments.

**Subtask 1b:** *Perform rolling experiments of MDAPCa2b cells using HBMEC and HUVECs on a parallel-plate flow chamber (1-4 months)*

MDAPCa2b prostate cancer cells were perfused through both parallel chamber and Fluxion biosciences flow chamber system. HUVECs were seeded as a monolayer and rolling velocity of MDAPCa2b cells were measured. At different shear stresses ranging from 0.5-4  $\text{dyn}/\text{cm}^2$ , rolling velocity of MDAPCa2b cells on IL-1 $\beta$ -stimulated HUVECs was found to range from 4.2-6  $\text{dyn}/\text{cm}^2$  (Figure 2). Unstimulated HUVECs used as a control did not show any rolling behavior. Interestingly, rolling behavior was completely abrogated in the presence of anti-E-selectin neutralizing antibody in IL-1 $\beta$ -stimulated HUVECs. A significant difference was observed in the rolling behavior between IL-1 $\beta$ -stimulated HUVECs and unstimulated HUVECs ( $p < 0.005$ ), and IL $\beta$ 1-stimulated HUVECs and IL-1 $\beta$  plus E-selectin neutralizing antibody HUVECs ( $p < 0.005$ ). These experiments confirmed that E-selectin in IL-1 $\beta$ -stimulated HUVECs leads to rolling behavior in MDAPCa2b cells.

**Subtask 1c:** *Assess the interactions of prostate circulating tumor cells from patients with the HBMEC and HUVECs (4-24 months)*

We obtained 15 ml blood from 4 metastatic prostate cancer patients. After CD45 depletion and anti-PSMA J591-488 labeling, we observed CTCs in 3 patients. Among these 3 patients, we observed tethering and firm adhesion of CTCs on stimulated HUVECs using parallel flow chamber system. For example, in patient #4, we observed 10 CTCs which were firmly adhered to IL-1 $\beta$ -stimulated HUVECs. Furthermore, in IL-1 $\beta$  plus neutralizing anti-E-selectin antibody, we found only 3 CTCs stuck to the endothelial cells at the end of flow. These experiments provide evidence that CTCs from prostate cancer patients interact with endothelial cells via E-selectin. These are ongoing experiments.

**Subtask 2a:** *Design and optimization of the immunofluorescent conditions using MDAPCa2b, PC3, U937, and SaOS2 cells (1-4 months)*

Tumor cells interact with E-selectin expressed on activated endothelial cells via a variety of E-selectin ligands (ESL). These ESLs express a unique carbohydrate motif, sLe<sup>x</sup>, which appears to be required for ESL binding. The chemokine receptor CXCR4 has also been reported to supporting transendothelial migration of prostate cells through bone marrow endothelial cells (6). We examined the expression pattern of both sLe<sup>x</sup> and CXCR4 on prostate cancer cells by immunofluorescence staining. Cells were also immunostained for PSMA and EpCAM to confirm a CTC of prostate origin. Antibody optimization experiments for these four protein markers were conducted using a set of positive and negative controls. MDAPCa2b cells were positive for all the 4 protein markers, while PC3 cells expressed only EpCAM and CXCR4 (Figures 3 and 4). KG1 cells, a leukemic cell line, were used as a positive control for sLe<sup>x</sup> and negative control for PSMA, and EpCAM (Figure 3). After establishing, the specificity of these 4 protein markers in cell lines, we also tested the specificity using normal blood from healthy donors spiked with prostate cancer cells (Figure 4). We found that there was no cross-reactivity between the primary antibodies using spiked poly blood mononuclear cells (PBMCs). These experiments are critical for the analysis of CTCs from prostate cancer patients.

### **Subtask 2b: *Enrichment of prostate CTCs using negative immunoselection by anti-CD45 magnetic beads (4-24 months)***

Blood from 3 metastatic prostate cancer patients was obtained. After the enrichment by CD45 immunomagnetic-bead depletion, obtained CTCs were cytopinned onto glass slides. CTCs were immunostained for PSMA and EpCAM to confirm a CTC of prostate origin. Immunostaining showed that the expression of sLe<sup>x</sup> was heterogenous in these 3 patients (Figure 5). Since these patients showed a very high number of CTCs; therefore, sLe<sup>x</sup> antigen expression was quantified. PBMCs from normal healthy donors, MDAPCa2b, and PC3 cells were used as sLe<sup>x</sup> controls to standardize the fluorescence intensity. PBMCs from healthy donors were markedly positive, MDAPCa2b cells were moderately positive and PC3 cells were negative for sLe<sup>x</sup> expression. Pie chart shows that the fluorescence intensity above 20 was considered as moderately positive; whereas, fluorescence intensity above 100 denoted a very high sLe<sup>x</sup> expression (Figure 6). Using the controls, we found that in these three patients, 89/203 (43.8%), 95/155 (61.3%), and 84/112 (75%) cells were moderately positive for sLe<sup>x</sup> while 5.4%, 1.9%, and 18.8%, cells respectively showed a very high expression of sLe<sup>x</sup> (Figure 6).

### **Progress with accomplishments and Discussion**

The metastatic cascade is believed to involve CTC adhesion with activated endothelial cells leading to CTC transmigration across the endothelium leading to metastasis. Several studies suggest that this interaction results in part from E-selectin ligand binding with E-selectin expressed on activated endothelial cells. In this progress report, we showed for the first time that CTCs derived from PCa patients demonstrate physical interactions with activated endothelial cells expressing E-selectin under physiological shear flow. These interactions were abrogated in the presence of E-selectin neutralizing antibody confirming that prostate CTCs interact with endothelial cells via E-selectin. These experiments raises the possibility of the presence of E-selectin ligands on the surface of prostate CTCs. To examine the E-selectin ligands, we immunostained CTCs for sLe<sup>x</sup> expression. The expression of sLe<sup>x</sup> and sLe<sup>a</sup>, carbohydrate motif present on glycoproteins such as E-selectin ligands is frequently associated with cancer progression and poor prognosis (7). In this report, we showed for the first time the expression of sLe<sup>x</sup> on CTCs derived from 3 metastatic prostate cancer patients. In addition, quantification of sLe<sup>x</sup> expression showed inter- and intra- variability in the patients.

Since, this is an ongoing project; therefore, we would be observing the interactions of prostate CTCs with endothelial cells in more patients.

### **Methodology**

**Prostate tumor cell-Endothelial interactions.** Parallel-plate flow chamber was used to assess the interactions between MDAPCa2b cells or CTCs derived from prostate cancer patients with human umbilical vein endothelial cells. MDAPCa2b cells or CTCs were perfused over confluent monolayers of HUVECs grown in a 35 X 10 mm (Corning Inc., Corning, NY) tissue culture dishes. HUVECs were stimulated with 50 ng/ml IL-1 $\beta$  (Peprotech, Rocky Hill, NJ) for 4 hrs. To confirm the E-selectin expression, cells were either fixed and immunostained with anti-human E-selectin monoclonal antibody (68-5H11, BD Pharmingen, San Jose, CA) or western blotting was performed using the same antibody. IL-1 $\beta$ -stimulated HUVECs treated with 30  $\mu$ g/ml neutralizing anti-human E-selectin 68-5H11 for 1 hr at 37°C incubator, and unstimulated HUVECs were used as controls. MDAPCa2b cells or CTCs were resuspended in HBSS/10mM HEPES/2mM CaCl<sub>2</sub>/0.5% HSA medium and infused into the parallel-flow chamber over HUVECs. MDAPCa2b cell rolling was assessed at different shear stresses 0.5-4 dyn/cm<sup>2</sup> during 3 different experiments. For rolling measurements in CTCs derived from prostate cancer patients, cells enriched by anti-CD45 immunomagnetic depletion and labeled with anti-PSMA J591-488, were perfused over HUVECs at 0.6 dyn/cm<sup>2</sup> shear stress. Rolling assay experiments were also performed using Fluxion Bioflux system (Fluxion Biosciences, San Francisco, CA). For these experiments, the

channel was first coated with fibronectin (50 µg/ml) kindly provided by Dr Tim Hla at Weill Cornell Medical College.  $1 \times 10^8$  cells/ 100 µl were used to coat the channel with a confluent monolayer of HUVECs. After overnight incubation, prostate tumor cells were perfused over IL-1 $\beta$  stimulated-HUVECs at different shear stress.

**Enrichment of prostate CTCs by anti-CD45 immunomagnetic-bead depletion.** To enrich for CTCs, PBMCs were isolated from peripheral blood by ficoll-density based centrifugation and subjected to negative selection for leukocytes using anti-CD45 immunomagnetic beads (Life Technologies, Grand Island, NY). Blood was collected in a sodium citrate tube containing 2.7 ml blood drawn from a metastatic prostate cancer patient. PBMCs were isolated, washed twice with 2% RPMI/4mM MgCl<sub>2</sub>/1mM CaCl<sub>2</sub> and incubated with anti-CD45 immunomagnetic beads for 20 min on a rotator. After incubation, 2% RPMI/4mM MgCl<sub>2</sub>/1mM CaCl<sub>2</sub> media was added and cells were kept on a magnet for 10 min. Cells were centrifuged at 400 g for 12 min and anti-PSMA J591-488 antibody was added for 40 min. After incubation, cells were resuspended in HBSS/10mM HEPES/2mM CaCl<sub>2</sub>/0.5% HSA medium and rolling assay was performed. For immunostaining experiments, after anti-CD45 immunomagnetic depletion, cells were resuspended in 500 µl of 2% RPMI medium and cytopspun onto positively charged glass slides (Thermo Scientific, Asheville, NC). Slides were stored at -20°C for subsequent immunostaining.

**Immunofluorescent staining of cell surface markers.** After anti-CD45 enrichment of PBMCs from CRPC patients, isolated cells cytopspun onto glass slides were fixed in 2% formaldehyde (Tousimis, Rockville, MD) for 20 min on a shaker, and washed 3X with PBS. Cells were blocked with 2% BSA for 1 h, and incubated with primary antibodies for anti-rat sLe<sup>x</sup> (1:10, BD pharmingen, San Jose, CA) or anti-rabbit CXCR4 (1:100, Novus Biologicals, Littleton, CO) for 1 h, and incubated with secondary antibodies (goat anti-rat AF594 and goat anti-rabbit dylight 405) for 1 h, incubated in AF-488 and AF-647 conjugated primary antibodies for anti-PSMA (humanized) and anti-EpCAM (mouse) and mounted on a coverslide with freshly prepared mowiol (Calbiochem, Billerica, MA). All incubations were performed at room temperature followed by 3X wash with PBS plus 0.5% BSA. For immunofluorescent staining, MDA, PC3, KG1 cells, and PBMCs obtained from normal healthy donors were used as negative and positive controls for different proteins. Control cells were seeded on BD cell-tak coated coverslips and processed similarly as patient samples. Quantification of sLe<sup>x</sup> expression data was obtained using Metamorph<sup>TM</sup> software (MDS Analytical Technologies, Sunnyvale, CA) and measuring the average fluorescence intensity per pixel. The background intensity, determined by selecting an area lacking CTCs, was subtracted from these values for each image. For these measurements, Z-stacks of the acquired images were used taken by LSM 700 Zeiss Observer.Z1 (Carl Zeiss, Microimaging Inc, Thornwood, NY).

#### 4. KEY RESEARCH ACCOMPLISHMENTS:

- Developed a novel way to identify the interactions between endothelial cells and CTCs derived from prostate cancer patients.
- Detected and confirmed that CTCs derived from prostate cancer patients interact with endothelial cells via E-selectin.

#### 5. CONCLUSION:

This research project is highly innovative and challenging. We have provided an evidence of the existence of interactions between endothelial cells and CTCs derived from metastatic prostate cancer

patients. These CTC-endothelial interactions occur via E-selectin, suggesting the presence of E-selectin ligands on the surface of prostate CTCs. These findings shed light on the potential pathway to prostate tumor cell metastasis. We propose to further determine and characterize the E-selectin ligands present on CTCs. In addition, we plan to examine the E-selectin ligands on CTCs which interacted with the endothelial cells versus the ones which did not show interactions with the endothelial cells. Determining the type of E-selectin ligands on CTCs would be exciting as potentially alternative splice variants of these E-selectin ligands might be responsible for the interactions with endothelial cells. These studies may provide new therapeutic targets for anti-metastatic therapies.

## 6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Gunjan Gakhar, Vicente Navarro, Guang Yu Lee, He Liu, Naveed Akhtar, Scott Tagawa, Neil H. Bander, Paraskevi Giannakakou, Michael R. King, David Nanus: *Prostate circulating tumor cells interact with E-selectin expressed on endothelial cells*. American Association for Cancer Research. April 6-10, Washington DC, 2013

Gunjan Gakhar, Vicente Navarro, Matthew Loftus, Neil H. Bander, Paraskevi Giannakakou, David Nanus. *Quadruple immunofluorescence protocol for identification of the metastatic potential of prostate CTCs*. American Association for Cancer Research. March 31-April 4, Chicago, IL, 2012.

## 7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

## 8. REPORTABLE OUTCOMES: Nothing to report

## 9. OTHER ACHIEVEMENTS: Nothing to report

## 10. REFERENCES:

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2010; 20: 169-177.

**11. APPENDICES:** Appendix I contains figures and figure legends for the results

**NOTE:**

**TRAINING OR FELLOWSHIP AWARDS:**

Training Opportunity at AACR

At 2013 AACR meeting, I was able to interact with Monica Burdick, Assistant Professor at Rush College of Biomedical Engineering. We had a 2 hr one-on-one meeting which was fruitful as we discussed ways to improve my research methodology.

Analytical and Quantitative Microscopy

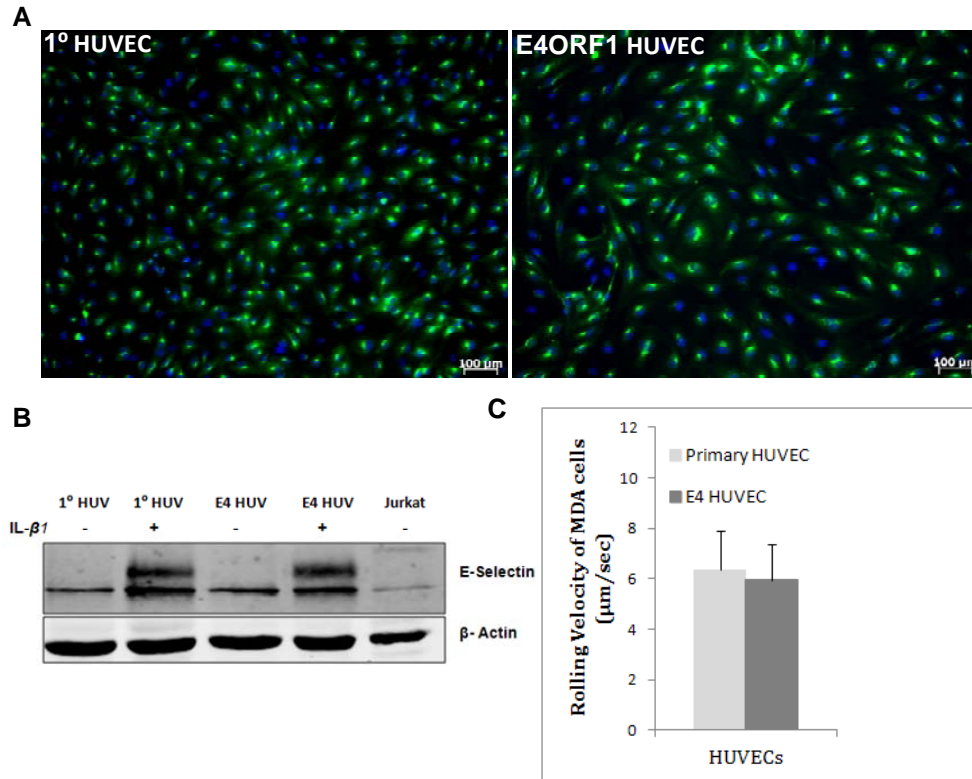
I have been selected for “Analytical and Quantitative Microscopy” workshop provided by Marine Biological Laboratory, Woods Hole, MA, USA from May 1 through May 10. This is a comprehensive and intensive course in light microscopy. This course provides a systematic and in-depth examination of the theory of image formation and application of video and digital methods for exploring subtle interactions between light and the specimen. This course emphasizes the quantitative issues that are critical to the proper interpretation of images obtained with modern wide-field and confocal microscopes. This workshop will be informative and useful for my ongoing project.

**COLLABORATIVE AWARDS:** Nothing to report

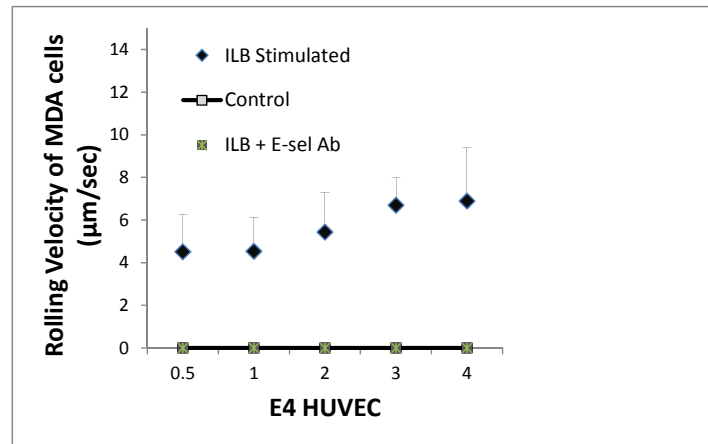
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## Appendix I



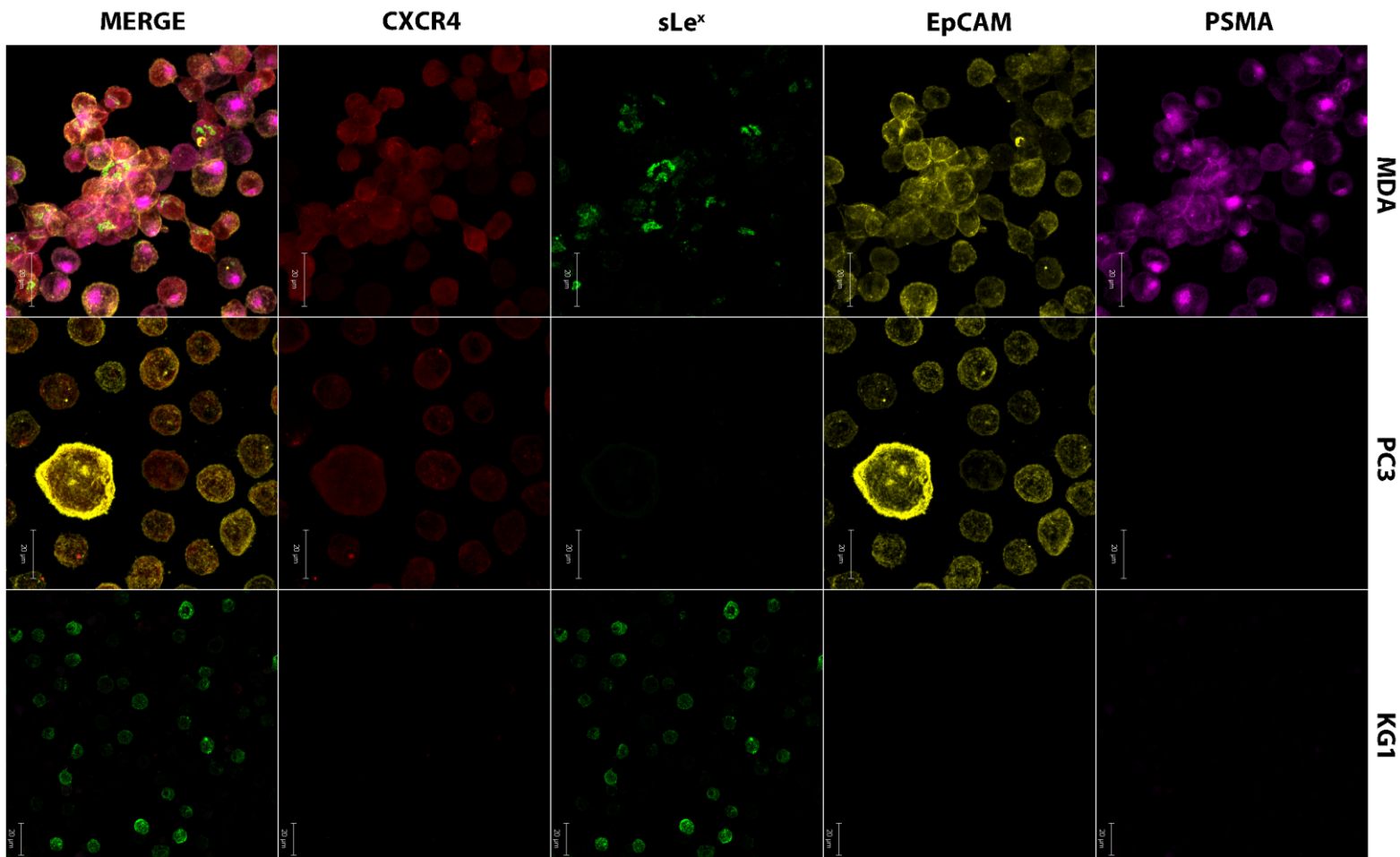
**Figure 1. E-selectin expression and its functional role in primary and E4-ORF1 human umbilical vein endothelial cells.** **A)** Immunofluorescence showing E-selectin expression in IL $\beta$ 1-stimulated 1<sup>0</sup>- and E4-ORF1 HUVECs. **B)** Western blot showing the E-selectin protein expression in primary (1<sup>0</sup>) and E4-ORF1 HUVECs. Cells were either stimulated with 50 ng/ml IL $\beta$ 1 or unstimulated. Jurkat cells were used as a positive control. Western blot is a representative of three independent experiments. **C)** Rolling velocity of MDAPCa2b (MDA) cells on IL $\beta$ 1-stimulated 1<sup>0</sup>- and E4-ORF1 HUVECs. Graph depicts Mean  $\pm$  SD.



**Figure 2. Rolling velocity of MDAPCa2b cells on stimulated E4-ORF1 HUVECs.**  $1 \times 10^6$  MDA cells were perfused over HUVECs. HUVECs were either stimulated with IL $\beta$ 1 or unstimulated or stimulated with IL $\beta$ 1 plus E-selectin neutralizing antibody. Graph shows Mean  $\pm$  SD.  $p < 0.005$  for both IL $\beta$ 1 and unstimulated HUVECs, or IL $\beta$ 1 plus E-selectin neutralizing antibody and IL $\beta$ 1-stimulated HUVECs.

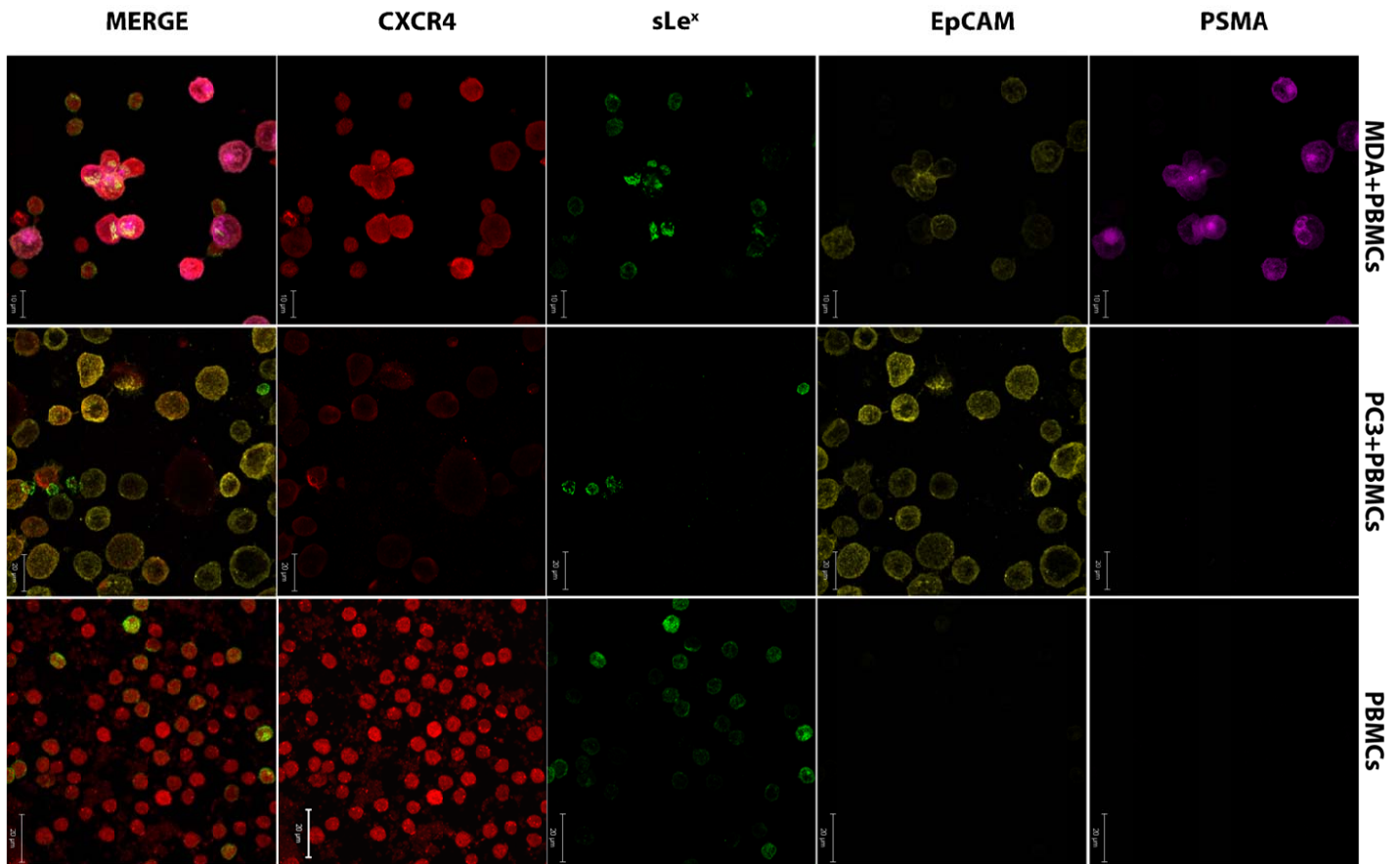
Patients	IL $\beta$ 1 HUVECs	IL $\beta$ 1 + E-sel HUVECs
1	5	0
2	4	1
3	0	0
4	10	3

**Table 1. CTC-Endothelial interactions using parallel flow chamber assay.** The number of CTCs that interacted with IL $\beta$ 1-stimulated HUVECs in the presence and absence of E-selectin neutralizing antibody.

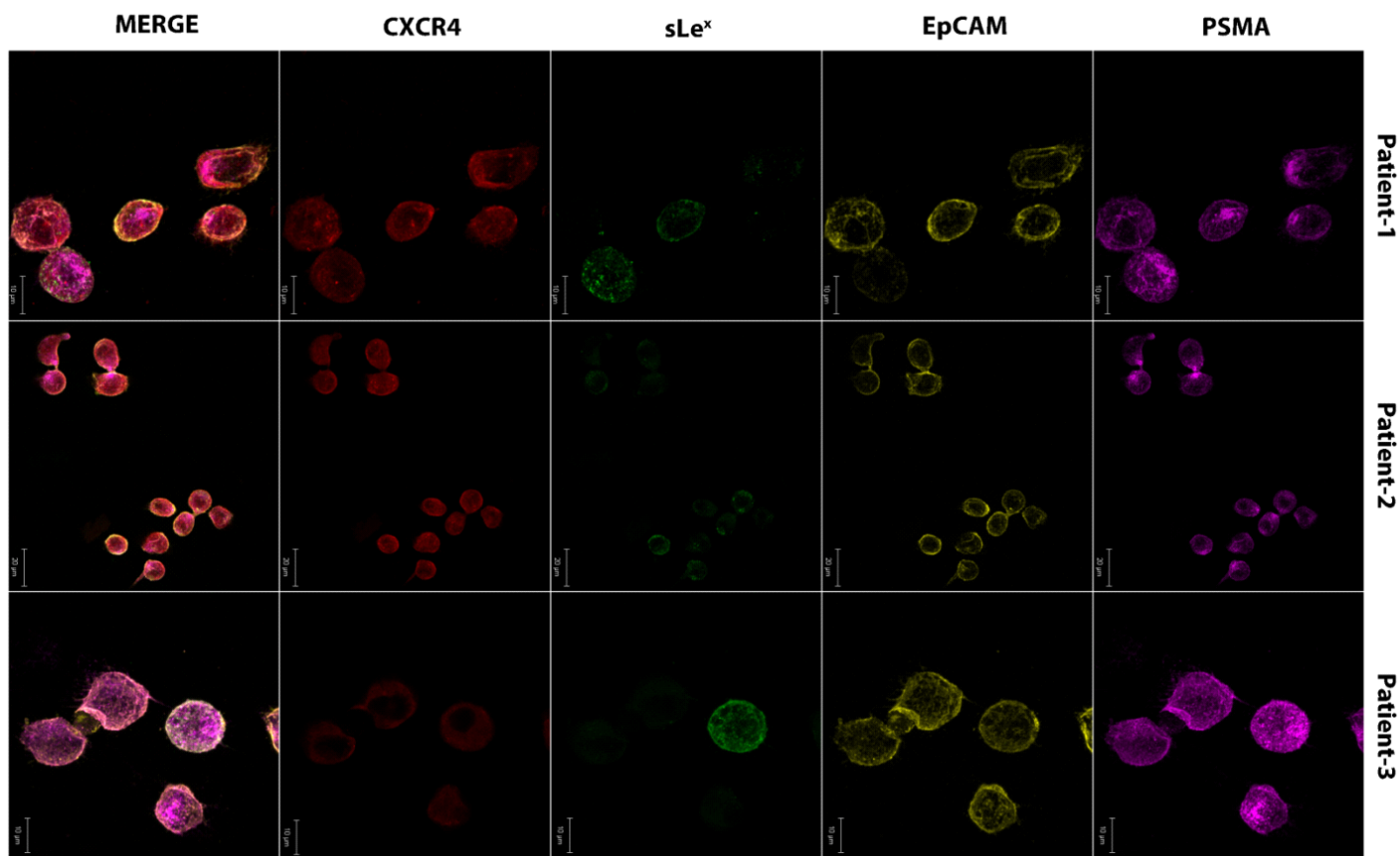


**Figure 3. Optimization of immunofluorescence staining of PSMA, EpCAM, sLe<sup>x</sup>, and CXCR4 proteins.** MDA, PC3, KG1 cells were used for the optimization experiments. Cells were seeded onto cell-tak coated 48 mm coverslips in a 48-well plate. Cells were fixed, blocked and incubated with a primary antibody for anti-rabbit CXCR4 and anti-rat sLe<sup>x</sup>. After washing with PBS, cells were put in respective secondary antibodies-anti-rabbit dylight 405 and anti-rat AF594. Cells were then incubated

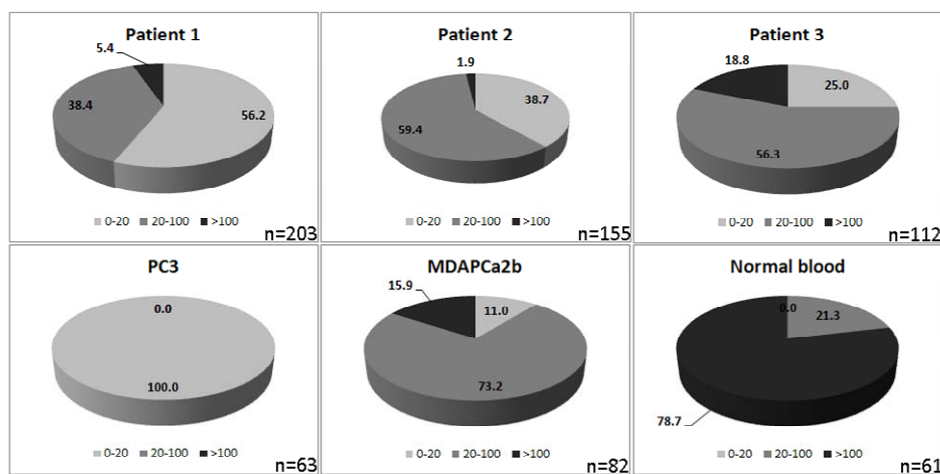
with conjugated primary antibodies- humanized mouse PSMA- AF488 and mouse EpCAM- AF647. PSMA= Magenta, EpCAM= Yellow, sLe<sup>x</sup>= Green, CXCR4= Red, and Merge shows all the colors.



**Figure 4. Immunofluorescence staining of normal healthy blood mixed with MDA, and PC3 cells.** Blood from normal healthy donors was spiked with either MDAPCa2b or PC3 cells. After ficoll-hypaque density centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated. Spiking experiments were conducted to observe the specificity of CTC markers (PSMA and EpCAM). After spiking, cells were seeded onto coverslips and stained as described in the methods and figure 3. PSMA= Magenta, EpCAM= Yellow, sLe<sup>x</sup>= Green, CXCR4= Red, and Merge shows all the colors.  
MDA=PSMA+, EpCAM+, sLe<sup>x</sup>+, CXCR4+  
PC3= PSMA-, EpCAM+, sLe<sup>x</sup>-, CXCR4+  
PBMCs= PSMA-, EpCAM-, sLe<sup>x</sup>+, CXCR4-



**Figure 5. Isolation of prostate CTCs from castrate-resistant prostate cancer patients using anti-CD45 immunomagnetic depletion.** 2.5 ml blood from metastatic prostate cancer patients were processed via ficoll density centrifugation and the PBMC fraction was collected. Immunomagnetic CD45 depletion was performed on the obtained PBMCs and the remaining cells were washed, cytopinned onto the slides. Slides were stained for PSMA, EpCAM, sLe<sup>x</sup>, and CXCR4. PSMA= Magenta, EpCAM= Yellow, HECA-452= Green, CXCR4= Red. All prostate CTCs expressed CXCR4, though the intensity of the CXCR4 protein varied both in the same patient sample and between the patient samples. sLe<sup>x</sup> expression was variable; therefore, quantified and depicted in Figure 6.



**Figure 6. Pie chart showing the percentage expression of sialyl Lewis X on human prostate CTCs.** PBMCs from normal healthy donor blood, MDA, and PC3 cells were used as high expressors, moderate, and negative controls for sLe<sup>x</sup> expression. Normal blood showed the highest expression. MDA cells have intermediate to high expression while PC3 cells are negative for the expression of sLe<sup>x</sup>. We observed variable expression in CTCs derived from three CRPC patients. n= number of CTCs. The legend indicates the fluorescence intensity values of sLe<sup>x</sup>. Nil to low sLe<sup>x</sup> expression = 0-20. Intermediate to Moderate expression = 20-100. High expression = >100.